DEVELOPMENT OF COUMARIN CONJUGATED CHITOSAN NANOFORMULATION TO CHECK ITS EFFICACY AGAINST POLYMICROBIAL SEPTICEMIA

Project report submitted to Sister Nivedita University in partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE

IN

MICROBIOLOGY

Submitted By

RAYA CHAKRABORTY

Registration ID:230010025261

Enrollment ID:2322211001002

Advisor

MR MAYUKH BOSE

HOD,Biotechnology

BOSE INFORMATICS

School of Health Science and Translational Research

Sister Nivedita University

Kolkata

June 2025

DEVELOPMENT OF COUMARIN CONJUGATED CHITOSAN NANOFORMULATION TO CHECK ITS EFFICACY AGAINST POLYMICROBIAL SEPTICEMIA

A PROJECT SUBMITTED BY

RAYA CHAKRABORTY

ENROLLMENT ID : 2322211001002

UNDER THE SUPERVISION OF

MR MAYUKH BOSE

BOSE INFORMATICS

53/F, FEEDER ROAD, BEEHIVE GARDEN,

BELGHORIA, KOLKATA, WEST BENGAL 700056

COMPLETION CERTIFICATE

BOSE INFORMATICS

c/o B Info Sol Pvt. Ltd.
Ph: 7439673045 / 7439733708
Website: www.boseinformatics.com
Email: informaticsbose@gmail.com
Address: 147/2 Feeder Road, Belgharia, Kolkata-700056

REF: BI/462/024/298

REG: B52200322000445

CERTIFICATE

OF COMPLETION

THIS CERTIFICATE IS PROUDLY PRESENTED TO

Raya Chakraborty

Who has completed a 90-days internship in DEVELOPMENT OF COUMARIN CONJUGATED CHITOSAN NANOFORMULATION TO CHECK ITS EFFICACY AGAINST POLYMICROBIAL SEPTICEMIA from 12th February 2025 to 20th May 2025 at our premises. We proudly present this certificate for your hard work, dedication, sincerity, perseverance and punctuality. We wish you all the best in your future endeavours.

20/05/2025
DATE OF ISSUE

TRAINING COORDINATOR

BOSE INFORMATICS

KOLKATA-700030 BOSE INFORMATICS WEST BENGAL

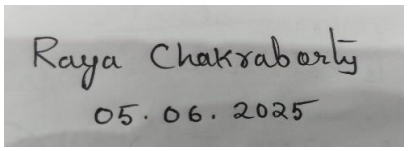
DECLARATION BY THE STUDENT

I hereby declare that the work which is being presented in this dissertation entitled "**DEVELOPMENT OF COUMARIN CONJUGATED CHITOSAN NANOFORMULATION TO CHECK ITS EFFICACY AGAINST POLYMICROBIAL SEPTICEMIA**" in partial fulfilment of the requirements for the award of the degree of Masters of Science in Microbiology is an authentic record of my own work carried out during the period from February 2025 to May 2025 under the supervision of Mr Mayukh Bose Department of Biotechnology,Bose Informatics.

I acknowledge that I have read and understood the UGC regulations, 2018 (*Promotion of Academic integrity and Prevention of Plagiarism in Higher Educational Institutions*).

I confirm that this dissertation has been checked for plagiarism using the plagiarism checking software Plagarism Checker. At the end of the dissertation, a copy of the summary report demonstrating similarities in content and its potential source (if any), generated online using plagiarism checking software is enclosed. I herewith confirm that the dissertation has been less than 10% similarity according to the plagiarism checking software's report and meets the UGC Regulations as well as plagiarism rules of Sister Nivedita University.

I further declare that no portion of the dissertation or its data will be published without the Institute's or supervisor's permission. I have not previously applied for any other degree or award using the topics and findings described in my dissertations.



(Signature of the students with Date)

Name of the Student- RAYA CHAKRABORTY

Registration no.- 230010025261

Department- Microbiology

SISTER NIVEDITA UNIVERSITY
সিস্টার নিবেদিতা বিশ্ববিদ্যালয়

DG Block - 1/2, Action Area I, New Town, Kolkata - 700156, West Bengal, India.

DEPARTMENT OF BIOTECHNOLOGY & DEPARTMENT OF MICROBIOLOGY
জৈব প্রযুক্তি বিভাগ ও অণুজীব বিদ্যা বিভাগ

TO WHOM IT MAY CONCERN

This is to certify that the project report titled "**DEVELOPMENT OF COUMARIN CONJUGATED CHITOSAN NANOFORMULATION TO CHECK ITS EFFICACY AGAINST POLYMICROBIAL SEPTICEMIA**", submitted by Ms. **RAYA CHAKRABORTY** (Registration No-**230010025261** Enrolment No-**2322211001002**) to the Department of Microbiology, School of Health Science and Translational Research at Sister Nivedita University, Kolkata, is a bona fide record of research conducted by her for the partial fulfillment of the requirements for the Master of Science degree.

Date:05.06.2025

Place: Newtown , Kolkata

Signature of Head of the Department

ACKNOWLEDGEMENT

This project would not have been possible without the direct and indirect contributions of many people. I take this opportunity to mention specifically some of them and extend my gratefulness to other well-wishers known and unknown.

I express a deep sense of gratitude towards my honorable guide Mr Mayukh Bose, HOD of BOSE INFORMATICS for his remarkable guidance, constant support and a gear of motivation that kept my spirit high even in extremely adverse conditions. I would also like to thank my lab partner Miss Rabina Pradhan for aiding me in this project and working really hard to see the fulfilment of this project. His dedication to this project increased its scientific value.

I like to express my deep sense of indebtedness to the management and staffs of BOSE INFORMATICS for giving me an opportunity to carry out my training.

I am also thankful to Mr Mayukh Bose for his constant feedback and support throughout the training.

Finally, I bow my head to my parents for their ruthless sacrifice, invariable endurance, unrestricted love and encouragement.

LIST OF FIGURES

Sl no	Fig No	Fig Name
1	1	Powdered Cinnamon taken in petri dish
2	2	Cinnamon powder is mixed with ethanol and heavily stirred
3	3	The solution is filtered using filter paper
4	4	After evaporation scrapping is done to take the black dust
5	5	Resultant coumarin blackish extract
6	6	Test tube showing the presence of coumarin in the sample
7	7	Test tube showing the presence of phenol in the sample
8	8	Thin Layer Chromatography Technique
9	9	Rf value obtained in TLC Technique
10	10	Nanoparticle preparation of chitosan
11	11	The UV range in spectrophotometer without the coating of the nanoparticle
12	12	Syringes and filters used to pass the nanoparticles
13	13	The peak showing in the spectrophotometer safter the coating of the drug
14	14	The graph of MIC polymicrobial set I with the dosage of 28.5 µl
15	15	The graph of MIC polymicrobial set II with the dosage of 55 µl
16	16	The graph of IC50 polymicrobial set I with the dosage of 62.8 µl

17	17	The graph of IC50 polymicrobial set II with the dosage of 78 µl
18	18	The graph of Fungal MIC with the dosage of 30 µl
19	19	The graph of Fungal IC50 with the dosage of 64 µl
20	20	A clear Circular Zone Of Inhibition(Fungal) is observed around the disc above in the figure. It means that the dosage of the coated sample (coumarin) could inhibit the fungal growth in the PDA agar plate
21	21	A clear Circular Zone Of Inhibition(Bacterial) is observed around the disc above in the figure. It means that the dosage of the coated sample (coumarin) could inhibit the bacterial growth in the LB agar plate

LIST OF TABLES

Sl No	Table Number	Table Name
1	1	The result of Set I Bcterial MIC
2	2	The result of Set II Bcterial MIC
3	3	The result of Set I IC50
4	4	The result of Set II IC50
5	5	The result of Fungal MIC
6	6	The result of Fungal IC50

ABBREVIATIONS

- TLC :Thin Layer Chromatography
- TPP: Sodium tripolyphosphate
- MIC:Minimum inhibitory concentration
- ZOI:Zone Of Inhibition
- PDA: Potato Dextrose Agar

ABSTRACT

Coumarin is a naturally occurring aromatic organic compound that is well known for its sweet smell and possible medicinal properties. It is mostly found in some types of cinnamon, especially Cinnamomum cassia. This study looks at how to get coumarin out of cinnamon bark, separate it, and clean it up using a set of well-known phytochemical methods. First, the bark of the cinnamon tree is dried and ground up. Then, it is put through a process called solvent extraction, which uses ethanol or methanol as the main solvent because these two substances are good at getting phenolic compounds out of the bark. After that, the crude extract is concentrated under low pressure with a rotary evaporator to make a semi-solid mass that is full of secondary metabolites. The next step is to use solvents with higher polarity, like hexane, chloroform, and ethyl acetate, to separate the liquids and selectively increase the amount of coumarin. This part goes through column chromatography, where silica gel is the stationary phase and a mix of hexane and ethyl acetate is the mobile phase. Thin-layer chromatography (TLC) is used to keep an eye on the fractions, and the ones that have coumarin in them are combined and cleaned up even more. Depending on how big the isolation is, recrystallization with methanol or preparative TLC can be used to purify the substance. Spectroscopic methods like UV-Vis spectroscopy can confirm the identity and purity of the coumarin that was isolated. Effective coumarin extraction and isolation are guaranteed by this meticulous process, which also emphasizes the significance of chromatographic methods and solvent selection in the purification of natural products. The biological activities of the purified coumarin and its possible uses in the food and pharmaceutical industries can then be investigated further.

KEYWORDS

Coumarin, Cinnamon,Thin Layer Chromatography,Enterococcus faecalis,Zone of Inhibition.

CONTENTS

- Introduction
- Review of Literature
- Materials and methods
- Results and discussions
- Conclusion
- Appendix
- References
- Plagiarism report

INTRODUCTION

The point of this study was to look at the impacts of coumarin derivatives with their isolation, physical and substance evaluation. Coumarins show a momentous exhibit of biochemical and pharmacological action. Coumarins comprising of intertwined benzene and α-pyrone rings are available in critical sums in plants, and more than 1300 coumarins have been identified from natural source. Derivatives of coumarins normally happen as auxiliary metabolites present in seeds, roots, and leaves of many plant species. Coumarins have an assortment of significant natural exercises, for example, anti-inflammatory, antioxidant, antiviral, antimicrobial and anticancer. Coumarins are shown to build focal sensory system activity.Coumarins are phenolic compounds broadly circulated in the plant kingdom. The isolation of coumarin was first detailed by Vogel in 1820.The name coumarin starts from a Caribbean word coumarou for the Tonka tree (Dipteryx odorata Wild, Leguminosae), what shares the trademark smell of these mixtures and was referred to organically at one time as Coumarouna odorata Aubl. Naturally happening coumarins, which are grouped by their benzopyran-2-one core, have been disconnected from various plants, especially individuals from the Apiaceae, Rutaceae, and Fucaceae, just as from certain genera of Leguminosae. The coumarins analogs (engineered or regular) are a fundamental part in the beauty care products and scents industry. Organically dynamic regular items have coumarin skeleton and utilized as a middle for the combination of bioactive heterocyclic mixtures which uncovered its antimicrobial, antifungal, mitigating, hostile to malignant growth, against tubercular, cell reinforcement, and anticoagulant properties. Hardly any manufactured mixtures with a coumarin ring have been accounted for to be dynamic against multi-drug safe (MDR) bacteria.Cinnamon a native flavor, having a place with the Lauracea family, is found in pretty much every family. Utilized chiefly as a seasoning specialist, it has been a significant constituent of our food since quite a while. For quite a while, our predecessors have been involving it as a solution for respiratory and stomach related diseases. Nonetheless, less is referred to about its advantageous impact as a cell reinforcement, calming, antilipidemic, antidiabetic, antimicrobial, and anticancer agent. the trial study show that cassia cinnamon contain follow measure of coumarin.Writing survey uncovers that coumarin (2-oxo-2H-chromene) and its subordinates address one of the most dynamic classes of heterocyclic mixtures which have a wide range of organic exercises: antitumor, antibacterial, antifungal, anticoagulant, cell reinforcement, and hostile to inflammatory.Coumarin (1,2-benzopyrone), a fragrant oxygen-containing heterocyclic compound, has different natural capacities. Past investigations have shown that coumarin and its subsidiaries display antibacterial movement against gram positive species S.aureus and gram negative species E.coli.

The ethylene moiety with carboxylic or tertiary amine groups, alkoxyimino groups, carbonyl groups, and hydroxamate groups is present at position C-3. Moreover, it is feasible to replace a benzyl group with a hydroxyl or halide group. In addition to Staphylococci and Enterococcus species, coumarins substituted with 3-ketone, 3-ester, and 3-alkoxyimino derivatives were thought to be exceptionally active against gram positive and gram negative strains. The effectiveness of the benzene rings substituted at the methylene-bis position as antibacterial agents was assessed; they were primarily effective against gram positive and gram negative microscopic organisms, such as E. coli and Staphylococcus aureus separately.

The antibacterial activity was most diminished when t-butyl was substituted at the para position. Gram positive and gram negative bacteria, such as S. aureus and E. coli, were most minimally inhibited by a methyl bunch substituted at the methylene-bis position. Coli. In comparison to the unsubstitute, at position C-4.

CHARACTERISTICAL ASPECTS OF COUMARIN:-

Coumarin is present in significant amounts in some varieties of cinnamon. Coumarins, which are also present in sweet clover, lavender oil, and Tonka beans, are abundant in Cassia cinnamon. It is highly soluble in ether and chloroform and only weakly soluble in water. 710 is its melting point. It tastes like vanilla caramel, has a sweet, fragrant smell, and has a mildly acidic character. Formula for molecules: C9H6O2 Weight in molecules: 146.145 g/mol.

Coumarin posses various activities as follows:-

- Anticancer activity: It has long been known that anti-malignant growth drugs interfere with the cell division process in order to damage the abnormally partitioning cell. Many mix treatments, in which a few cytotoxic specialists are included in the treatment system, offer better results with fewer toxic secondary effects because they are meticulously controlled to allow recovery of normal, but not harmful, cells from drug openness. These medications are effective and cytotoxic, and as a result, they exhibit extreme aftereffects, particularly on normal multiplying tissues like the hematopoietic framework. The best outcomes for patients with malignant growth are currently provided by chemotherapy, radiotherapy, and medical procedures combined. Therapy blends have also been successfully used for certain disease types, such as Hodgkin's lymphoma, testicular disease, and various leukemias. It is possible to use coumarins to treat secondary effects in addition to malignant growth.
- Antifungal activity: In comparison to other infectious strains such as Aspergillus niger (NCIM No. Penicillium Chrysogenum (NCIM No. 1196). Curvularia Lunata (NCIM No. 723) and 723. 1131). Standard drugs such as amphotericin B, miconazole, and fluconazole were used to test the antifungal activity. After 48 hours, the absorbance at 410 nm was measured to assess the inhibition of cell development. The plates were brooded at 37 C for all microorganisms. The MIC was identified as the creature's least focus-repressing development. As a negative or dissolvable control, DMSO was used. Separate tests using DMSO arrangements alone revealed no action against any microbial strains, which may help to explain any effects of DMSO on natural screening. The mixtures selected for least inhibitory focus studies are those exhibiting promising antifungal activity. Measurements at 128, 64, 32, 16, 8, 4, 2, 1, and 0point 5 lg/mL foc show that the concentration is still in the air.
- Antibacterial activity: Although coumarin has very little antibacterial activity, coumarin products with long hydrocarbon replacements, like ostruthin and ammoresinol, are thought to be the most effective treatment for Gram-positive bacteria, including Staphylococcus aureus and Bacillus megaterium.Newly combined mixtures were tested for antimicrobial susceptibility against bacterial strains called viz. Gram-positive Aureus Staphylococcus (ATCC No. Micrococcus Luteus (ATCC No. 29737). 398), Cereus Bacillus (ATCC No. Gram-negative Escherichia coli (NCIM No. 6630) and S. Pseudomonas fluorescens (NCIM No. 2256). As well as Flavobacterium Devorans (ATCC No. 2173). 10829), one at a time, to determine the

least inhibitory focus. All examples were prepared in three-fold sequential twofold weakenings in microtiter plates and vaccinated Med Chem Res 123 with a suitably pre-arranged cell suspension to achieve the required introductory focus. sequential deterioration.

REVIEW OF LITERATURE

Since of their wide extend of chemical composition and natural movement, common items have long been utilized as a premise for medicate advancement and revelation. A normally happening phenolic compound that's a part of the benzopyrone family is coumarin. It is recognized for a assortment of organic and pharmacological qualities, such as anticoagulant, antimicrobial, anti-inflammatory, antioxidant, and anticancer impacts, and for having a unique sweet scent that's reminiscent of naturally cut roughage or vanilla. Coumarin is now a compound of incredible intrigued within the nourishment and pharmaceutical businesses since of its wide extend of applications and basic simplicity.A common zest and conventional medicine, cinnamon is made from the internal bark of trees within the class Cinnamomum (family Lauraceae). Among the diverse species, Chinese cinnamon, or Cinnamomum cassia, is well-known for having a tall coumarin substance. In spite of the fact that coumarin includes to cinnamon's wonderful fragrance and conceivable restorative benefits, over the top utilization can have negative wellbeing impacts, such as hepatotoxicity and carcinogenicity, especially when taken in large amounts over an expanded period of time. As a result, administrative offices just like the European Nourishment Security Specialist (EFSA) have built up limits on the sum of coumarin that can be devoured, which calls for correct expository and preliminary strategies to degree and, in the event that vital, partitioned this substance. For both logical and commonsense employments, the method of coumarin extraction, separation, and refinement from cinnamon is fundamental. Regularly, extraction employments natural solvents to viably extricate coumarin from the plant framework. Whereas refinement strategies, such as recrystallization or chromatographic division (e.g., thin-layer chromatography or column chromatography), are pivotal for getting coumarin in its immaculate shape appropriate for advance pharmacological thinks about or detailing, separation centers the target compound utilizing techniques like liquid-liquid apportioning or solid-phase extraction.In arrange to extricate, separate, and decontaminate coumarin from Cinnamomum cassia, this proposal will systematically explore and move forward the methods.

In arrange to optimize yield and virtue whereas diminishing negative impacts on the environment and human wellbeing, the think about will concentrate on choosing the proper solvents and methods. The comes about are expected to back quality control in items inferred from cinnamon, reinforce security controls with respect to the utilization of coumarins, and progress a more extensive understanding of normal compound preparing.

Coumarin is a fragrant organic compound found in many plants, especially in Chinese cinnamon (Cinnamomum cassia). It is used in the pharmaceutical, food, and cosmetic industries, but its use is monitored because high doses can harm the liver.To extract coumarin, researchers often use organic solvents like ethanol and methanol through methods like maceration or Soxhlet extraction. Newer methods, such as supercritical CO_2 extraction and microwave-assisted extraction, have been developed to use less solvent and save time while increasing yield.Once extracted, coumarin needs to be separated from other compounds in cinnamon, like cinnamaldehyde. This is typically done using techniques like column chromatography and thin-layer chromatography, with high-performance liquid

chromatography (HPLC) becoming popular for its speed and accuracy.For purification, recrystallization using ethanol or ethyl acetate is common to produce pure coumarin crystals. Preparative HPLC is also used for high-purity coumarin suitable for various applications. The final product's identity and purity are confirmed using methods like UV-Vis, IR, and NMR spectroscopy.

Polymic infections includes simultaneous presence of two or more microorganisms - batteries, the fungi or virus - contribute to symption of the seriousness of illness. These infizations are often more resistant to treatment and generally are found in wounds, biofilm and immunocomromated. The boom in antimicrobal resistance complicated the strategies of treatment. 2 The rental metabolites in antimicrobal therapy

Side metabolites (eg, alkalids, terpsomi, macogo, fenbontians but biological vows by plants, fungi and microrganes. Many have an antimicrobal activity with the spectrum to disturb the cell walls in disturbing or promoting an oxidative stress in pathogens examples: Curcumin: Antibassiteri, antifurn, antias

Berberina: break the recurrence of backered adn

QUERCETIN: Symary with antibiotics

However, his low solability, its unwanted and their poor availability limit their clinical applications.Nanopolimder as medication management systems

Nanopolimers as plege, peg, chitosan and desquitted for the drug administration because of their biodeadive molecule haidective. Nanocarkers improve destination distribution, controlled release and the celling absorion of an antimicial agents .

AGAINS OF NANOPOLIMERS:

Increasing penetration in biophilma

Prolonged systemic circulation

Decreate toxicity for host host

4 Strategies conjugation for secondary metabolites with nanopolimers

To overcome the sidewalks' pharmacinetic boundaries, researchers opposes always always with nanopolimers. Techniques include:

Cash link (eg chemistry EDC / NHS)

Capsulation in Nanoparticularly in the clean

Surface adsorption through electrostic interactions

Examples of studying:

Chitosan Nanoparticules filled with curcuminated improvement in antibacterial activity against S. Aureus and gram Aeruginosa. The nanoparticles combined plata rid of the collections of bactorids and enhanced bacteria collections Access the polacrobal infections.Conjugated nanopolimers tried to promise in the fight against polemicial infections:

BiofilM Urbanza: Nanopolimers easy the deeper penetration in complex biofils produced by multiple species. The synergist action: secondary metabolites often have improved effects when associated with nanoparticles, increasing their mixed communities against mixed mixed communities. Target specification: Nanopartices functionality allows the target's specific pathogen or tissue.

MATERIALS AND METHODS

Extraction, Isolation and purification of secondary metabolites :-

ISOLATION:

Fig 1: Powdered Cinnamon taken in petri dish.

1. Powdered cinnamon is taken and mixed with 99% ethanol in ratio of 1:9 and heavily stirred in a beaker for 15-20 mins.

Fig 2: Cinnamon powder is mixed with ethanol and heavily stirred.

2. Filtered the solution and kept the filtrate (Ethanol solubilize the coumarin along with other phenols and flavins in it).

Fig 3: The solution is filtered using filter paper.

3. Phenol is removed by heating it in air oven at 60 degree celcius for time period per 5g of cinnamon for 2-3 hours.

EXTRACTION AND PURIFICATION:

1. After the ethanol solvent evaporation,the extract is now devoid of phenol and other less boiling point materials,forming blackish extracts of small dusts.

Fig 4: After evaporation scrapping is done to take the black dust.

Fig 5: Resultant coumarin blackish extract

2. The blackish extract has some water soluble contents that is eluted out by mixing 10ml of distilled water and mixed them in magnetic stirrer for half an hour.
3. Then the mixture is evaporated to black dust.
4. Resultant coumarin solvent forms from black dust by mixing it with 50% ethanol mixed in mortar and pestle.
5. Now NaOH is added to make the pH to 8 to avoid precipitation.

Biochemical Characterization of Coumarin :-

TEST FOR COUMARIN:

- 3 ml of 10% NaOH is taken and mixed in 2mg of blackened extract.
- Fade yellow colour is obtained which shows the presence of coumarin.
- In the figure given below, yellow colour formed shows the presence of coumarin in the sample.

Fig 6: Test tube showing the presence of coumarin in the sample.

TEST FOR PHENOL:

- 5% Fecl3 is made on the base of excess NH4OH.
- Black colour is obtained which shows that phenol is present in the sample.
- Hence in the figure given below, black colour is formed which confirms the presence of phenol in my coumarin sample.

Fig 7: Test tube showing the presence of phenol in the sample.

DETECTION:

- UV-Vis Spectrophotometer: 300 nm-0.35

  240 nm-0.25

Hence my sample has given the UV range of 0.23 in 240nm in sphectrophotometer..So, Coumarin is detected.

PURITY ASSESMENT:

- Thin Layer Chromatography:
  Silica is powdered using mortar pestle and distilled water is added then dried into the air oven forming a thin layer. In TLC ,Rf value or Retention Factor explain the distance between the sample and the solvent. So Rf factor is basically defined as the distance travelled by the sample divided by distance travelled by the solvent.

Fig 8: Thin Layer Chromatography Technique.

For TLC 40% chloroform and 60% ethanol is used. The Rf value of coumarin in a 40% chloroform mobile phase for TLC is typically around 0.5-0.7.

According to my sample(coumarin)-

Distance travelled by sample=3.2 cm
Distance travelled by solvent= 6.6 cm
Therefore Rf value= 3.2/6.6 cm
=0.48 cm.

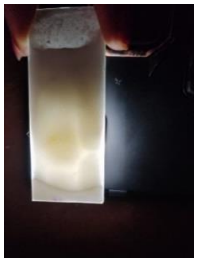


Fig 9: Rf value obtained in TLC Technique.

The Rf value given and the Rf value obtained from my sample is same so we got to know that purity is maintained by doing the TLC.

Nano Particle Preparation of Chitosan :-

Fig 10: Nanoparticle preparation of chitosan

The chitosan nanoparticle doped indocyanine green(CNPs-ICG) will be prepared.

- Briefly,50 mg of chitosan powder(ACROS Organic uk) with a low molecular weight(1-3 kDa) will be dissolved in 50ml deionized distilled water.
- After 1 hour at room temperature,the solution will be placed on the magnetic stirringand 1% acetic acid solution will be added dropwise within 30 mins to create the homogeneous. chitosan solution.
- Sodium tripolyphosphate [TPP 0.5% (W/V), 2mL] as a crosslinking agent will be added dropwise and the compound will be stirred 30 meus.
  (According to the Yongmei and Yumen study, TPP will strengthen the matrix of the CNPs.The mechanical strength of the CNPs matrix will increase by increasing the no of crosslinks formed between CNPs and TPP and these nanoparticles become stronger and harder).
- 2.0 mg/mL ICG will then be added to the prepared chitosan solution and mixed via a magnetic mixer for 10 meus at 1000 rpm, în at pH 4.8.
- Eventually, this solution will be centrifuged at 10,000 ppm for 30mins.
- The collected sediment CNPS-ICG will be typ lyophilized for further experiments.
- The entire chitosan nanoparticle solution is passed through a 0.22 micron syringe filter to make sure particles below the range of 200 nm passes through the solution after which the entire solution is scanned from 200-800 nm in spectrophotometer which should give a peak concluding nanoparticles are formed.

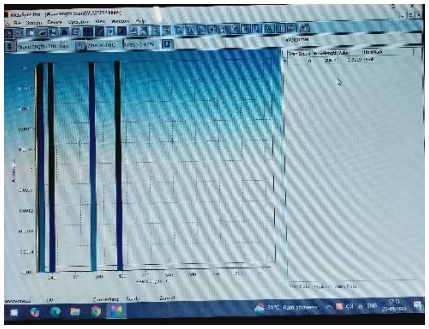


Fig 11: The UV range in spectrophotometer without the coating of the nanoparticle

Fig 12: Syringes and filters used to pass the nanoparticles.

- Once the nanoparticles are coated with drugs again the solution is scanned through a UV Sphectrophotometer from 200-800 nm and again the peak is observed.

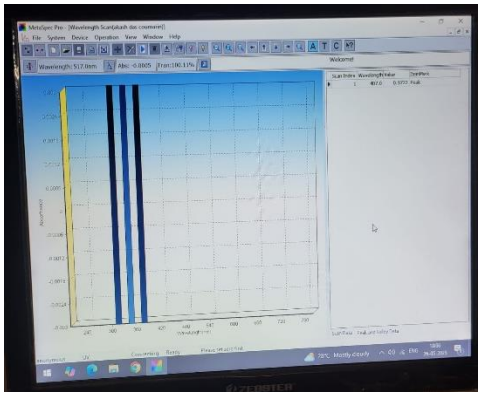


Fig 13: The peak showing in the spectrophotometer safter the coating of the drug.

Now in comparision we can see the peak before and the peak after has subsequential changes.So, the after peak has the much higher wavelength or higher absorbance than the only chitosan nanoparticle which indicates the successful coating of the drug on the nanoparticles.

Minimum Inhibitory Concentration (MIC):-

The MIC (Minimum Inhibitory Concentration) is a method to find the lowest amount of an antimicrobial agent that stops the visible growth of a microorganism in a controlled setting. This method is commonly used for testing how sensitive microbes are to antibiotics. To conduct MIC testing, different concentrations of antibiotics are prepared and tested against a standardized amount of bacteria, usually in LB or on agar media.

Historically, evidence for an infectious disease causative agent was obtained by fulfilling Koch's postulates, which require the isolation of the microbe responsible for infection from the site of infection, cultivation in a laboratory, and reproduction of the initial infection by introducing pathogen back into the host. They support the identification of microbial species present at the moment of infection by excluding the presence of other microorganisms in the course of the disease. The theories are based on the assumption that secondary microbial species with low abundances play no role in inciting the disease. However, it is becoming most obvious, even to the most credulous, that most, if not all, diseases are not monomicrobial in nature and manifestation.

Specifically, at this point we believe that many infections initiate—as remain—polymicrobial, similar to most wound infections and CAUTI, or they initiate as polymicrobial inoculation into the infection site.

Indeed, since it was recognized for some time, the role that polymicrobial bacterial communities can play in driving specific disease states—such as otitis media and periodontitis—is relatively well characterized. However, there is emerging consensus that some traditionally considered monomicrobial diseases, such as urinary tract infections, are also driven by other organisms present at lower titers during active infection and by the microbial consortium at the time of infection. The formation of biofilms-often protected environments in which microbial organisms can replicate-is another common feature in chronic and/or recurrent infections. For polymicrobial culture, three or four types of bacteria able to metabolize on their own on one culture are needed with no certain hurdles required. Whereas most bacteria in nature either coexist peacefully with other bacteria or are thoroughly inhibited by them due to a certain bacteriotoxin, in the laboratory environment one unique bacterium is isolated, cultivated and then introduced back with the rest. The primary purpose of testing for antibiotic activity in polymicrobial cultures is to find evidence of broad-spectrum antibiotic action of a synthesised component or phytochemical. While a certain bacteria will cause a different disease depending on the source of contamination and whether the bacterium exists in multicolonial form, it must be able to be infectious at that number. But the goal of exploiting that component in the future is to become an antibiotic to a number of bacteria, meaning that if a drug is made with it, its effectiveness must count to be cost-effective and help the economy of medicines.

Polymicrobial Cohort Preparation

In order to make polymicrobial cultures, 2 different sets of microorganisms were made. The 3 different sets were as follows:

Set 1: Enterococcus faecalis, Listeria monocytogenes, Serratia marcescens

Set 2: Pseudomonas aeruginosa, Campylobacter jejuni, Listeria monocytogenes

Set 1 was made on the basis that both the Enterococcus and Listeria species secretes cytolysin toxin that form pores on the cell membrane of Gram-positive bacteria like Staphylococcus aureus and Lactococcus lactis. Serratia species was made a part of this set as it secretes ShlA toxin that inhibits Gram negative bacteria.

Set 2 was made on the basis that Campylobacter and Listeria survive together in livestock and are the causal agents of several diseases in animals. Pseudomonas forms biofilm in case of environmental stress conditions. Formation of biofilm helped the other two microorganisms along with Pseudomonas to survive together

Microbial colonies from plates of 2 different bacteria for each set was taken using an inoculation loop and then inoculated into liquid LB media of 60 ml and then kept in an incubator at 37°C for 24 hours. After the incubation period, those cultures were again spread on specific media of those three bacteria and the microbes were allowed to grow at 37°C. Next day, each bacterial plate contained 95% of the specific bacteria which confirmed the growth of the three microbes in the poly microbial cohort. Also, staining confirmed the presence of the three microbes in the cohort. The cohort was prepared according to the survivability of the microbes with each other and hence we checked the survivability according the gram character, toxins released and rate of competitive inhibition.

The steps of doing MIC are as follows:

- Bacterial plate preparation-

  1.Media of 45 ml(with agar) was prepared and it was heated in microwave oven
  2. Waited until it cooled down properly.
  3.Poured them in 5 different plates (As 2 sets of polymicrobial bacterial was used and each set has 3 different types of bacteria and 1 bacteria common in both the sets)
  4.Added a drop of bacterial culture from the mother/father culture.
  5.Spreading was done.
  6.Sealed the plate using tape to avoid contamination and kept in incubator for the overnight growth of the bacterial culture.

- Inoculation-

  1.For inoculation a LB Broth was prepared.
  2. A minimal loop from the polymicrobial set of bacteria was isolated from the agar media

3.Then that loop was transferred into the LB media and kept in shaker for further mixing and the proper growth of the bacterial culture in the LB media.

- After some hours, OD was taken into the spectrophotometer and waited until it reaches 0.55-0.6 which indicates a specific bacterial growth.
- After a while when OD reached 0.6, 3 ml was kept for the standard solution and the drug(nanocoated coumarin sample) was added to the LB media to check whether the sample drug could inhibit the bacterial growth.The dosage of drug added was calculated as (C1)(V1)=(C2)(V2) which is shown below.
- 3 different OD of the standard solution and the sample solution has been recorded in the interval of 1 hour to see the difference of growth in the sample solution comparing it with the standard solution.

The polymicrobial set of bacteria that has been used for set I are- Enterococcus faecalis, Listeria monocytogenes, Serratia marcescens.

C1(Initial conc) = 10,500 µg/ml

V1(Drug volume) = ?

C2(Final conc) = 100 µg/ml

V2 (Standard solution) = 3ml

(C1)(V1)=(C2)(V2)

10,500*V1=100*3

V1=28.5 µl

The polymicrobial set of bacteria that has been used for set II are- Pseudomonas aeruginosa, Campylobacter jejuni, Listeria monocytogenes.

C1(Initial conc) = 10,500 µg/ml

V1(Drug volume) = ?

C2(Final conc) = 192.5 µg/ml

V2 (Standard solution) = 3ml

(C1)(V1)=(C2)(V2)

10,500*V1=192.5*3

V1=55 µl

Half Maximal Inhibitory Concentration(IC50) :-

Basically IC50 is defined as the how much the drug could inhibit a specific biological reaction by 50%.In case of IC50 value, the drug is more effective because it inhibits the growth at half concentration.

Like MIC, in IC50 too 2 sets of polymicrobial bacteria are used for the dosage of drug to show the inhibition at 50% concentration. The dosage has been calculated as (C1)(V1)=(C2)(V2) as shown below-

For IC 50 (Set I)-

C1(Initial conc) = 10,500 µg/ml

V1(Drug volume) = ?

C2(Final conc) = 220 µg/ml

V2 (Standard solution) = 3ml

(C1)V1=(C2)(V2)

10,500*V1=220*3

V1=62.8 µl

For IC 50 (Set II)-

C1(Initial conc) = 10,500 µg/ml

V1(Drug volume) = ?

C2(Final conc) = 260 µg/ml

V2 (Standard solution) = 3ml

(C1)V1=(C2)(V2)

10,500*V1=260*3

V1=78 µl

Zone Of Inhibition(ZOI)-

It is defined as the area where the bacteria or fungus could not grow due to a presence of drug that inhibites the growth rate of a particular bacteria or fungus

We have done ZOI by using polyset 1 MIC bacteria .

Enterococcus faecalis, Listeria monocytogenes, Serratia marcescens

The steps of doing Bacterial ZOI are as follows:

- First we prepared LB agar media and heated .
- Then poured the agar media into a petri dish.
- Then wait for sometime so that it cool down properly.
- Then we make a well in the middle of the plate and add polyset bacteria culture approx 100µl.
- We kept the plates in incubator for 24hours
- Next day we saw the ZOI in the plate
- Then the diameter of the ZOI was measured

Also ZOI emphasizes on the fungi Aspergillus niger.So, fungal ZOI is a clear area around an antifungal drug on a petri dish, showing where fungal growth is stopped. This zone shows how well the drug works. Larger zones mean stronger antifungal activity.

The steps of doing fungal ZOI are as follows:

- At first PDA media was prepared
- Then inoculated with the fungi Aspergillus niger.
- A well was cut using a tip in that petri dish and in that well 100 µl of the drug sample(nanocoated coumarin sample) was added.
- Incubated for 24 hours
- The diameter of ZOI was measured around the well. The larger ZOI indicates the greater antifungal activity.

RESULT AND DISCUSSION

Table 1: The result of Set I Bcterial MIC.The dosage given is compared to the standard. Starting OD=0.59

Standard	Dosage
0.66	0.58
0.79	0.58
0.84	0.61

Fig 14: The graph of MIC polymicrobial set I with the dosage of 28.5 µl

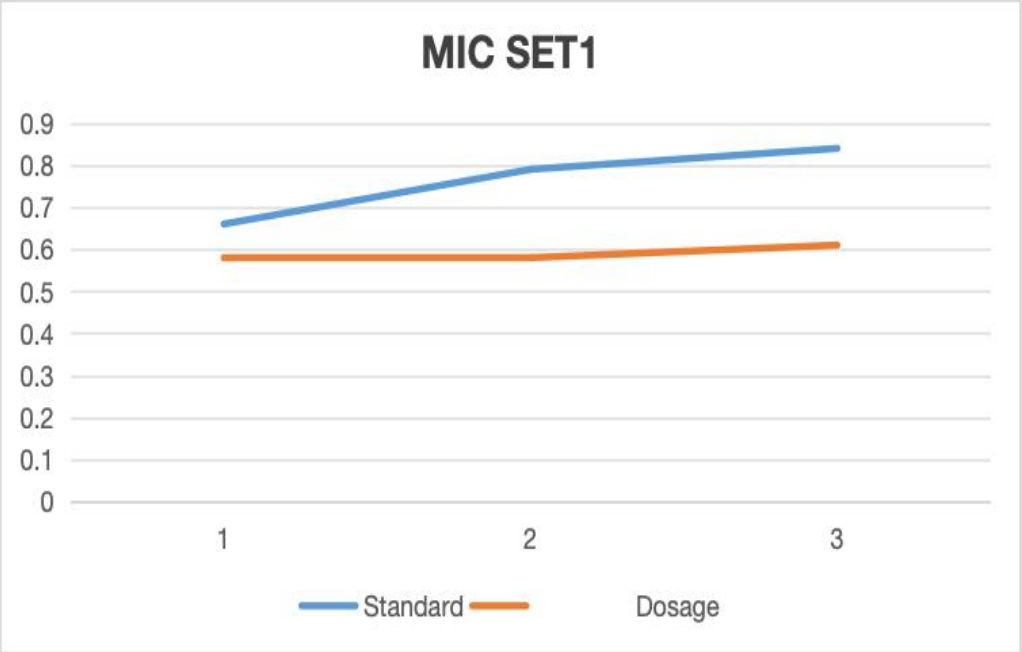


Table 2: The result of Set I MIC.The dosage given is compared to the standard

Starting OD=0.61

Standard	Dosage
0.72	0.62
0.76	0.64
0.80	0.67
0.82	0.65

Fig 15: The graph of MIC polymicrobial set II with the dosage of 55 µl

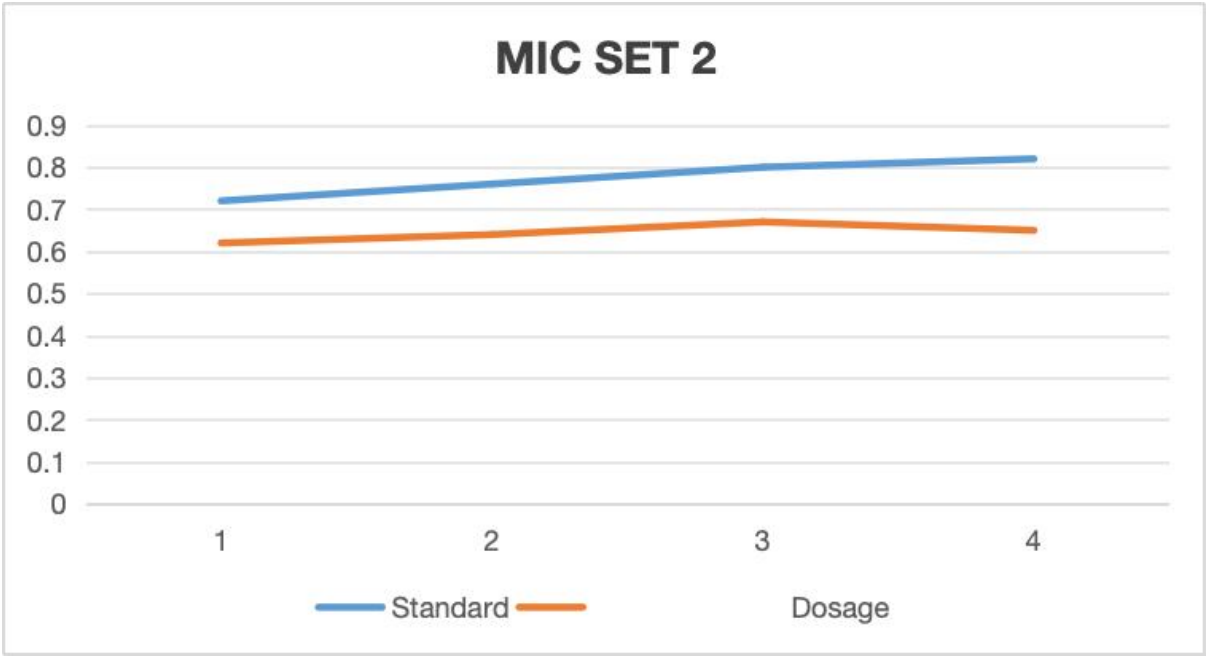


Table 3: The result of Set I IC50.The dosage given is compared to the standard.

Starting OD=0.54

Standard	Dosage
0.50	0.44
0.50	0.38
0.45	0.33

Fig 16: The graph of IC50 polymicrobial set I with the dosage of 62.8 µl

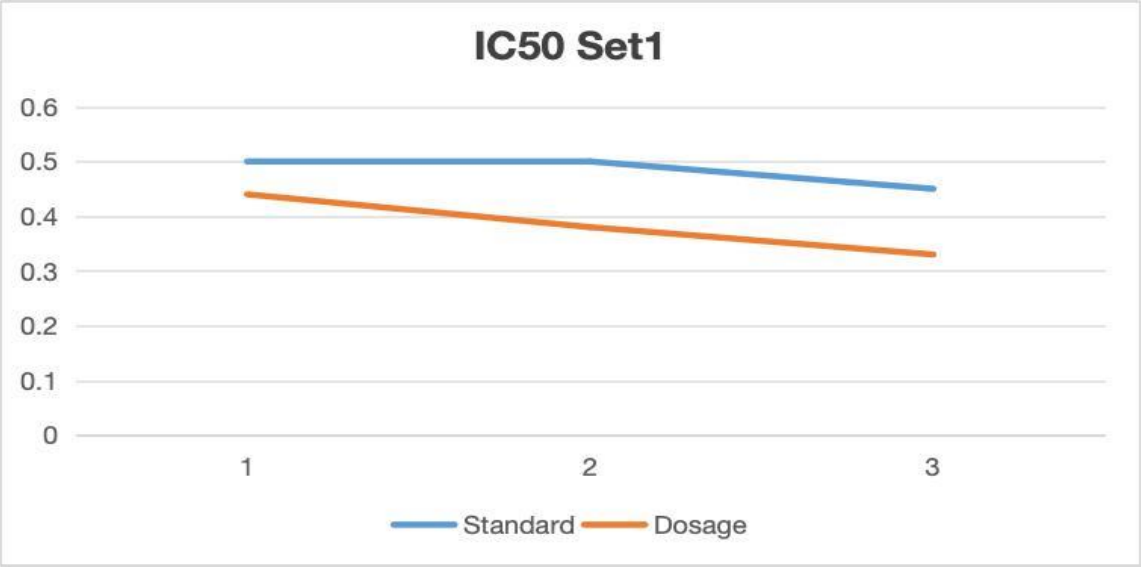


Table 4: The result of Set II IC50.The dosage given is compared to the standard.

Starting OD=0.54

Standard	Dosage
0.53	0.47
0.55	0.43
0.49	0.38

Fig 17: The graph of IC50 polymicrobial set II with the dosage of 78 µl

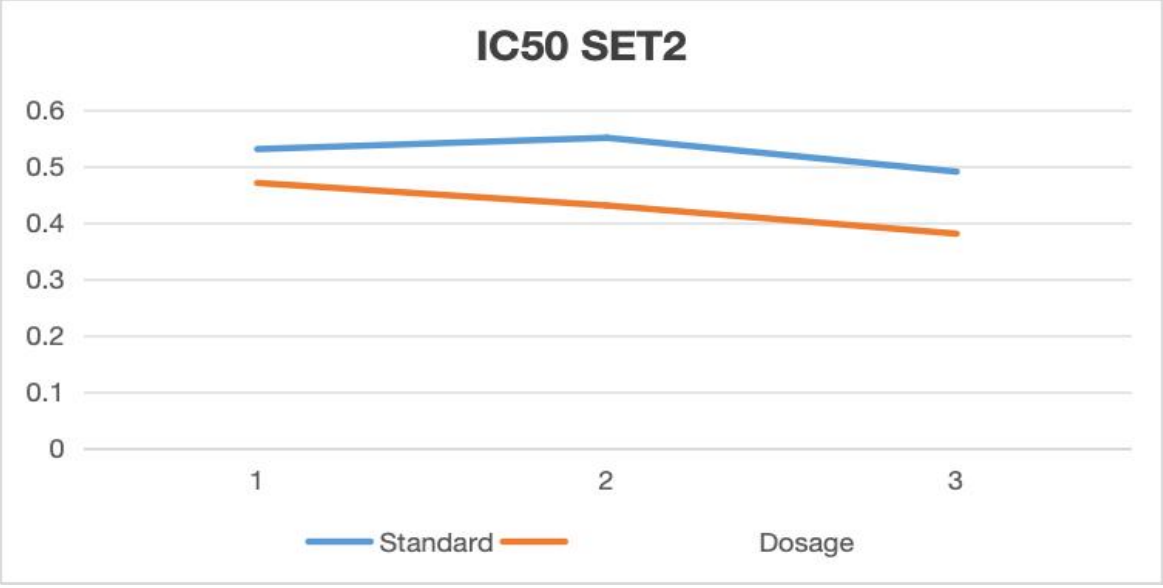


Table 5: The result of Fungal MIC.The dosage given is compared to the standard.

Starting OD=0.61

Standard	Dosage
0.67	0.58
0.72	0.62
0.76	0.65

Fig 18: The graph of Fungal MIC with the dosage of 30 µl

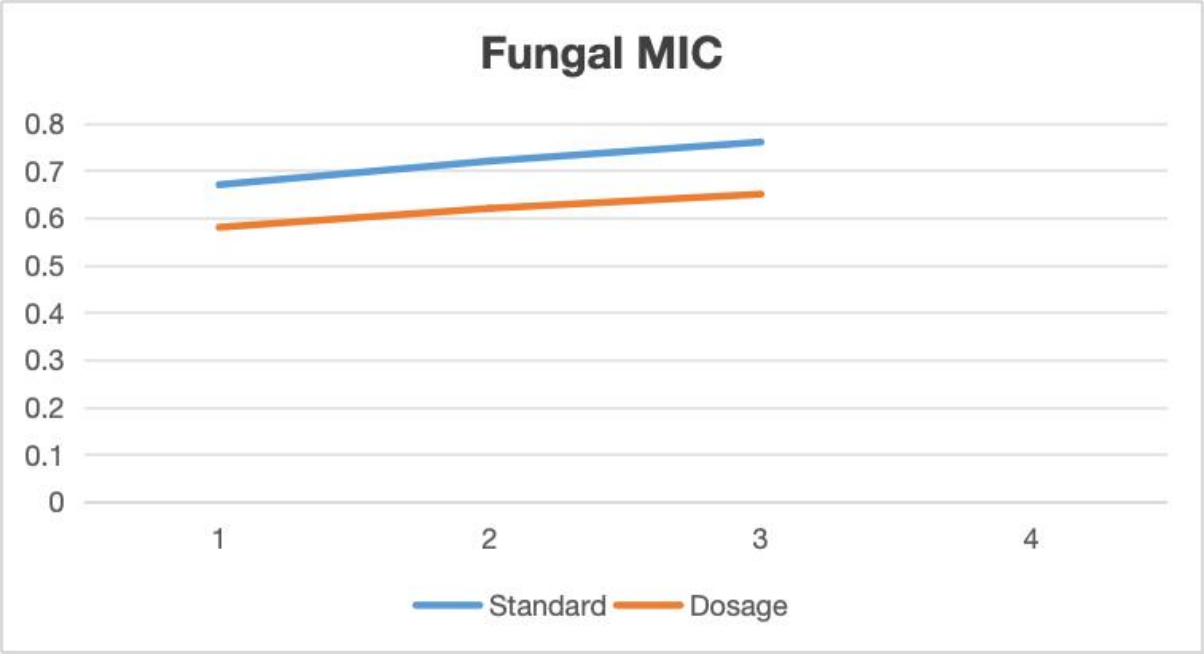
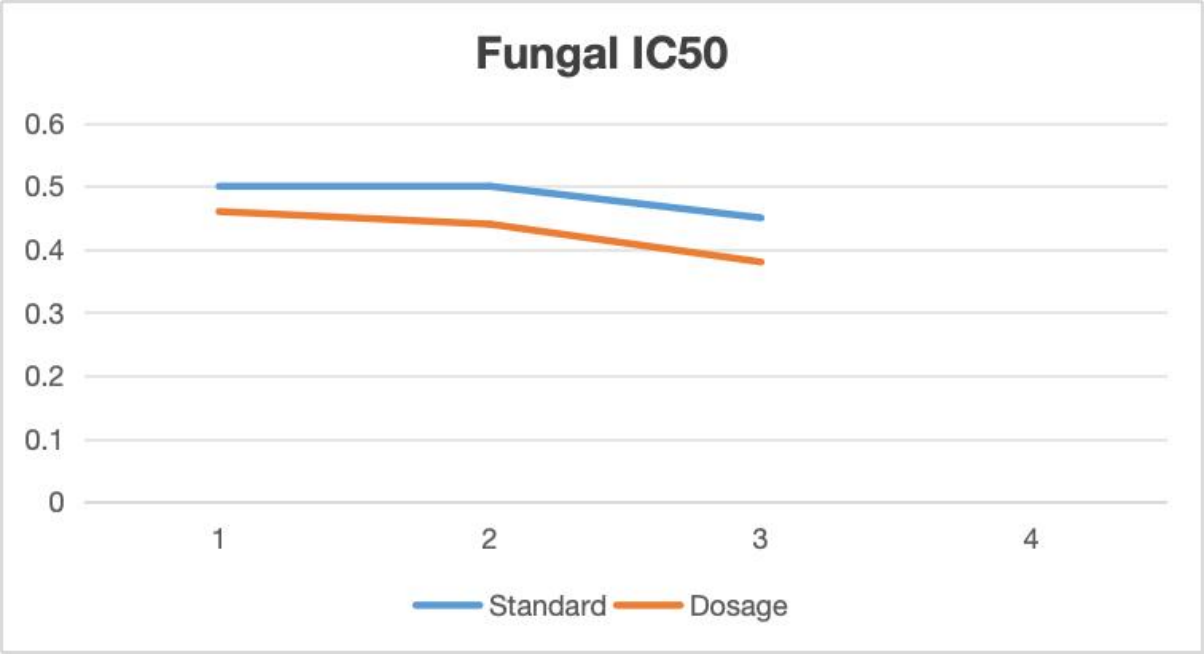


Table 6: The result of Fungal IC50.The dosage given is compared to the standard.

Starting OD=0.56

Standard	Dosage
0.50	0.46
0.50	0.44
0.45	0.38

Fig 19: The graph of Fungal IC50 with the dosage of 64 µl



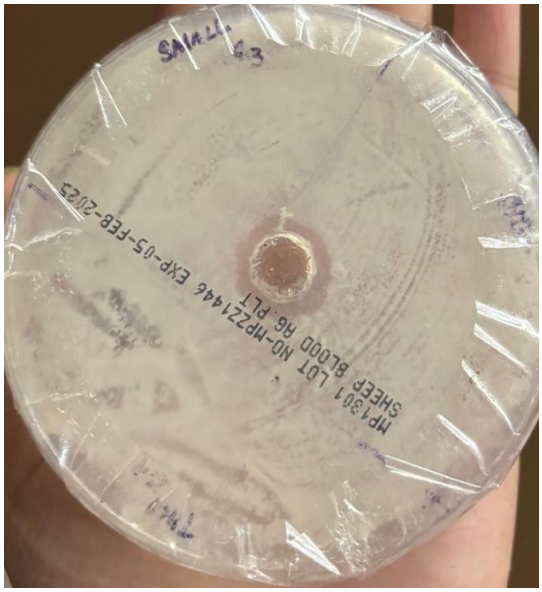


Fig 20: A clear Circular Zone Of Inhibition(Fungal) is observed around the disc above in the figure. It means that the dosage of the coated sample (coumarin) could inhibit the fungal growth in the PDA agar plate.

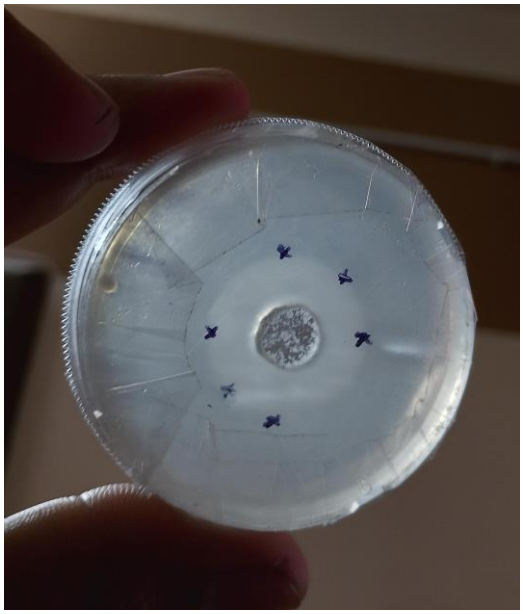


Fig 21: A clear Circular Zone Of Inhibition(Bacterial) is observed around the disc above in the figure. It means that the dosage of the coated sample (coumarin) could inhibit the bacterial growth in the LB agar plate

CONCLUSION

It could be concluded from the above data and graphs that Coumarin has a antibacterial property present in them and could inhibit the growth of bacteria and fungi.It works against both gram positive and gram negative bacteria.Coumarin is a potent natural antimicrobial agent.Coumarin also gives development of many alternative antimicrobial therapies.So, overall this study lead us to the path of many new upcomings of the advantages of coumarin as an important antimicrobial agent and also encourages continued research in pharma and clinical development.

APPENDIX

1. Introduction
2. Materials And Methods
   - Extraction, Isolation and purification of secondary metabolites
   - Biochemical Characterization of Coumarin
   - Nano Particle Preparation of Chitosan
   - Minimum Inhibitory Concentration (MIC)
   - Half Maximal Inhibitory Concentration(IC50)
   - Zone Of Inhibition(ZOI)
3. Result And Discussion
4. Conclusion

REFERENCES

- https://doi.org/10.3390/antibiotics13010071
- https://doi.org/10.31018/jans.v13i1.2482
- https://doi.org/10.1016/j.chroma.2005.03.023
- https://doi.org/10.1016/j.chroma.2005.04.041
- https://doi.org/10.1556/jpc.18.2005.3.11
- DOI NO: 10.5281/zenodo.6405608
- https://doi.org/10.1016/j.foodchem.2022.133029

PLAGARISM REPORT

Plagiarism Checker X - Report

Originality Assessment

10%

Overall Similarity

Date: May 29, 2025 (01:32 PM)
Matches: 478 / 5033 words
Sources: 18
Verify Report:
Scan this QR Code

Remarks: Low similarity detected, consider making necessary changes if needed.

9.0.4- WML 4 FILE-THIESIS.DOCX

DEVELOPMENT OF COUMARIN CONJUGATED CHITOSAN NANOFORMULATION TO CHECK ITS EFFICACY AGAINST POLYMICROBIAL SEPTICEMIA

A PROJECT SUBMITTED BY

RAYA CHAKRABORTY

ENROLLMENT ID : 2322211001002

UNDER THE SUPERVISION OF

MR MAYUKH BOSE

BOSE INFORMATICS

53/F, FEEDER ROAD, BEEHIVE GARDEN,

BELGHORIA, KOLKATA, WEST BENGAL 700056

ACKNOWLEDGEMENT

This project would not have been possible without the direct and indirect contributions of many people. I take this opportunity to mention specifically some of them and extend my gratefulness to other well-wishers known and unknown.

I express a deep sense of gratitude towards my honorable guide Mr Mayukh Bose, HOD of BOSE INFORMATICS for his remarkable guidance, constant support and a gear of motivation that kept my spirit high even in extremely adverse conditions. I would also like to thank my lab partner Miss Rabina Pradhan for aiding me in this project and working

really hard to see the fulfilment of this project. His dedication to this project increased its scientific value.

like to express my deep Isense of indebtedness to the management and staffs of BOSE INFORMATICS for giving me an opportunity to carry out my training.

I am also thankful to Mr Mayukh Bose for his constant feedback and support throughout the training.

Finally, I bow my to my parents for their headruthless sacrifice, invariable endurance, unrestricted love and encouragement.

ABSTRACT

16 Coumarin is a naturally occurring aromatic organic compound that is well known for its sweet smell and possible medicinal properties. It is mostly found in some types of cinnamon, especially Cinnamomum cassia. This study looks at how to get coumarin out of cinnamon bark, separate it, and clean it up using a set of well-known phytochemical methods. First, the bark of the cinnamon tree is dried and ground up. Then, it is put through a process called solvent extraction, which uses ethanol or methanol as the main solvent because these two substances are good at getting phenolic compounds out of the bark. After that, the crude extract is concentrated under low pressure with a rotary evaporator to make a semi-solid mass that is full of secondary metabolites. The next step is to use solvents with higher polarity, like hexane, chloroform, and ethyl acetate, to

separate the liquids and selectively increase the amount of coumarin. This part goes through column chromatography, where silica gel is the stationary phase and a mix of hexane and ethyl acetate is the mobile phase. Thin-layer chromatography (TLC) is used to keep an eye on the fractions, and the ones that have coumarin in them are combined and cleaned up even more. Depending on how big the isolation is, recrystallization with

methanol or 2 TLC can be used to preparativepurify the substance. Spectroscopic methods like UV-Vis spectroscopy can confirm the identity and purity of the coumarin that was isolated. Effective coumarin extraction and isolation are guaranteed by this meticulous process, which also emphasizes the significance of chromatographic methods and solvent selection in the purification of natural products. The biological activities of the purified coumarin and its possible uses in the food and pharmaceutical industries can then be

investigated further.

KEYWORDS

Coumarin, Cinnamon,Thin Layer Chromatography,Enterococcus faecalis,Zone of Inhibition.

INTRODUCTION

1 The point of this study was to look at the impacts of coumarin derivatives with their

isolation, physical and substance evaluation. coumarins show a momentous exhibit of biochemical and pharmacological action. Coumarins [3] comprising of intertwined benzene and α-pyrone rings are available in critical sums in plants, and more than 1300 coumarins

[1]

have been identified from natural source. Derivatives of coumarins normally happen as auxiliary metabolites present in seeds, roots, and leaves of many plant species. Coumarins have an assortment of significant natural exercises, for example, antiinflammatory, antioxidant, antiviral, antimicrobial and anticancer. Coumarins are shown to build focal sensory system activity.Coumarins are phenolic compounds broadly circulated in the plant kingdom. The isolation of coumarin was first detailed by Vogel in 1820.The name coumarin starts from a Caribbean word coumarou for the Tonka tree (Dipteryx odorata Wild, Leguminosae), what shares the trademark smell of these mixtures and was referred to organically at one time as Coumarouna odorata Aubl. Naturally happening coumarins, which are grouped by their benzopyran-2-one core, have been disconnected from various plants, especially individuals from the Apiaceae, Rutaceae, and Fucaceae, just as from certain genera of Leguminosae. The coumarins analogs (engineered or regular) are a fundamental part in the beauty care products and scents industry. Organically dynamic regular items have coumarin skeleton and utilized as a middle for the combination of bioactive heterocyclic mixtures which uncovered its antimicrobial, antifungal, mitigating, hostile to malignant growth, against tubercular, cell reinforcement, and anticoagulant properties. Hardly any manufactured mixtures with a coumarin ring have been accounted for to be dynamic against multi-drug safe (MDR) bacteria.Cinnamon a native flavor, having a place with the Lauracea family, is found in pretty much every family. Utilized chiefly as a seasoning specialist, it has been a significant constituent of our food since quite a while. For quite a while, our predecessors have been involving it as a solution for respiratory and stomach related diseases. Nonetheless, less is referred to about its advantageous impact as a cell reinforcement, calming, antilipidemic, antidiabetic, antimicrobial, and anticancer agent. the trial study show that cassia cinnamon contain follow measure of coumarin.Writing survey uncovers that coumarin (2-oxo-2Hchromene) and its subordinates address one of the most dynamic classes of heterocyclic mixtures which have a wide range of organic exercises: antitumor, antibacterial, antifungal, anticoagulant, cell reinforcement, and hostile to inflammatory.Coumarin (1,2-benzopyrone), a fragrant oxygen-containing heterocyclic compound, has different natural

negative strains.

capacities. Past investigations have shown that coumarin and its subsidiaries display antibacterial movement against gram positive species S.aureus and gram negative species E.coli. The ethylene moiety with carboxylic or tertiary amine groups, alkoxyimino groups, carbonyl groups, and hydroxamate groups is present at position C-3. Moreover, it is feasible to replace a benzyl group with a hydroxyl or halide group. In addition to Staphylococci and Enterococcus species, coumarins substituted with 3-ketone, 3-ester, and 3-alkoxyimino derivatives were thought to be exceptionally active against

The effectiveness of the benzene rings substituted at the methylene-bis position as antibacterial agents was assessed; they were primarily effective against gram positive and gram negative microscopic organisms, such as E. coli and Staphylococcus aureus separately. The antibacterial activity was most diminished when t-butyl was substituted at the para position. Gram positive and gram negative bacteria, such as S. aureus and E. coli, were most minimally inhibited by a methyl bunch substituted at the methylene-bis position. Coli. In comparison to the unsubstitute, at position C-4.

CHARACTERISTICAL ASPECTS OF COUMARIN:-

Coumarin is present in significant amounts in some varieties of cinnamon. Coumarins, which are also present in sweet clover, lavender oil, and Tonka beans, are abundant in Cassia cinnamon. It is highly soluble in ether and chloroform and only weakly soluble in water. 710 is its melting point. It tastes like vanilla caramel, has a sweet, fragrant smell, and has a mildly acidic character. Formula for molecules: C9H6O2 Weight in molecules:

146.145 g/mol.

Coumarin posses various activities as follows:-

- Anticancer activity: It has long been known that anti-malignant growth drugs interfere with the cell division process in order to damage the abnormally partitioning cell. Many mix treatments, in which a few cytotoxic specialists are included in the treatment system, offer better results with fewer toxic secondary effects because they are meticulously controlled to allow recovery of

normal, but not harmful, cells from drug openness. These medications are effective and cytotoxic, and as a result, they exhibit extreme aftereffects, particularly on normal multiplying tissues like the hematopoietic framework. The best outcomes for patients with malignant growth are currently provided by chemotherapy, radiotherapy, and medical procedures combined. Therapy blends have also been successfully used for certain disease types, such as Hodgkin's lymphoma, testicular disease, and various

2 It is possible to leukemias.use coumarins to treat secondary effects in addition to malignant growth.

8 strains such as Aspergillus niger (NCIM No. Penicillium Chrysogenum (NCIM No. • Antifungal activity: In comparison to other infectious

1196). Curvularia Lunata (NCIM No. 723) and 723. 1131). Standard drugs such as amphotericin B, miconazole, and fluconazole were used to test the antifungal activity. After 48 hours, the absorbance at

1 The plates were brooded at 37 C for all microorganisms. 410 nm was measured to assess the inhibition of cell development.

The MIC was identified as the creature's least focus-repressing development. As a negative or dissolvable control, DMSO was used. Separate tests using DMSO arrangements alone revealed no action against any microbial strains, which may help to explain any effects of DMSO on natural screening. The mixtures selected for least inhibitory focus studies are those exhibiting promising antifungal activity. Measurements at

128, 64, 32, 16, 8, 4, 2, 1, and 0point 5 lg/mL foc show that the concentration is still in the air.

 Antibacterial activity: Although coumarin has very little antibacterial activity, coumarin products with long hydrocarbon replacements, like ostruthin and ammoresinol, are thought to be the most effective treatment for Gram-positive bacteria, including Staphylococcus aureus and Bacillus megaterium.Newly combined mixtures were tested for antimicrobial susceptibility against bacterial

strains called viz. Gram-positive Aureus Staphylococcus (ATCC No. Micrococcus Luteus (ATCC No. 29737). 398), Cereus Bacillus (ATCC No.

Gram-negative Escherichia coli (NCIM No. 6630) and S. Pseudomonas fluorescens (NCIM No. 2256). As well as Flavobacterium Devorans (ATCC No. 2173). 10829), one at a time, to determine the least inhibitory focus. All examples were prepared in three-fold sequential twofold weakenings in microtiter plates and vaccinated Med Chem Res 123 with a suitably pre-arranged cell suspension to achieve the required introductory focus. sequential deterioration.

REVIEW OF LITERATURE

Since of their wide 6 of chemical composition and extendnatural movement, common items have long been utilized as a premise for medicate advancement and revelation. A normally happening phenolic compound that's a part of the benzopyrone family is coumarin. It is recognized for a assortment of organic and pharmacological qualities, such as anticoagulant, antimicrobial, anti-inflammatory, antioxidant, and anticancer impacts, and for having a unique sweet scent that's reminiscent of naturally cut roughage or vanilla. Coumarin is now a compound of incredible intrigued within the nourishment and pharmaceutical businesses since of its wide extend of applications and basic simplicity.A common zest and conventional medicine, cinnamon is made from the internal bark of trees within the class Cinnamomum (family Lauraceae). Among the diverse species, Chinese cinnamon, or Cinnamomum cassia, is well-known for having a tall coumarin substance. In spite of the fact that coumarin includes to cinnamon's wonderful fragrance and conceivable restorative benefits,

over the top utilization can have negative wellbeing impacts, such as hepatotoxicity and carcinogenicity, especially when taken in large amounts over an expanded period of time. As a result, administrative offices just like the European Nourishment Security Specialist (EFSA) have built up limits on the sum of coumarin that can be devoured, which calls for correct expository and preliminary strategies to degree and, in the event that vital, partitioned this substance. For both logical and commonsense employments, the method of coumarin extraction, separation, and refinement from cinnamon is fundamental. Regularly, extraction employments natural solvents to viably extricate coumarin from the plant framework. Whereas refinement strategies, such as recrystallization or chromatographic division (e.g., thin-layer chromatography or column chromatography), are pivotal for getting coumarin in its immaculate shape appropriate for advance pharmacological thinks about or detailing, separation centers the target compound utilizing techniques like liquid-liquid apportioning or solid-phase extraction.In arrange to extricate, separate, and decontaminate coumarin from Cinnamomum cassia, this proposal will systematically explore and move forward the methods.

In arrange to optimize yield and virtue whereas diminishing negative impacts on the environment and human wellbeing, the think about will concentrate on choosing the proper solvents and methods. The comes about are expected to back quality control in items inferred from cinnamon, reinforce security controls with respect to the utilization of coumarins, and progress a more extensive understanding of normal compound preparing.

Coumarin is a fragrant organic in [17] compound found in many plants, especially Chinese cinnamon (Cinnamomum cassia). It is used [18] in the pharmaceutical, food, and cosmetic

industries, but its use is monitored because high doses can harm the liver.To extract coumarin, researchers often use organic solvents like ethanol and methanol through methods like maceration or Soxhlet extraction. Newer methods, such as supercritical CO_2 extraction and microwave-assisted extraction, have been developed to use less solvent and save time while increasing yield.Once extracted, coumarin needs to be separated from other compounds in cinnamon, like cinnamaldehyde. This is typically done using techniques like column chromatography 11 high-performance liquid chromatography (HPLC) and thin-layer chromatography, with becoming popular for its speed and accuracy.For purification, recrystallization using ethanol or ethyl acetate is common to produce pure coumarin crystals. Preparative HPLC is also used for high-purity coumarin suitable for various applications. The final product's identity and purity are confirmed using methods like UV-Vis, IR, and NMR spectroscopy.

Polymic infections includes simultaneous presencemicroorganisms - 2 of two or more batteries, the fungi or virus - contribute to symption of the seriousness of illness. These infizations are often more resistant to treatment and generally are found in wounds, biofilm and immunocomromated. The boom in antimicrobal resistance complicated the strategies of treatment.

2 The rental metabolites in antimicrobal therapy

Side metabolites (eg, alkalids, terpsomi, macogo, fenbontians but biological vows by plants, fungi and microrganes. Many have an antimicrobal activity with the spectrum to disturb the cell walls in disturbing or promoting an oxidative stress in pathogens examples:

Curcumin: Antibassiteri, antifurn, antias

Berberina: break the recurrence of backered adn

QUERCETIN: Symary with antibiotics

However, his low solability, its unwanted and their poor availability limit their clinical applications.Nanopolimder as medication management systems

Nanopolimers as plege, peg, chitosan and desquitted for the drug administration because of their biodeadive molecule haidective. Nanocarkers improve destination distribution, controlled release and the celling absorion of an antimicial agents .

AGAINS OF NANOPOLIMERS:

Increasing penetration in biophilma

Prolonged systemic circulation

Decreate toxicity for host host

4 Strategies conjugation for secondary metabolites with nanopolimers

To overcome the sidewalks' pharmacinetic boundaries, researchers opposes always always with nanopolimers. Techniques include: Cash link (eg chemistry EDC / NHS)

Capsulation in Nanoparticularly in the clean

Surface adsorption through electrostic interactions

Examples of studying:

Chitosan Nanoparticules filled with curcuminated improvement in antibacterial activity against S. Aureus and gram Aeruginosa. The nanoparticles combined plata rid of the collections of bactorids and enhanced bacteria collections Access the polacrobal infections.Conjugated nanopolimers tried to promise in the fight against polemicial infections:

BiofilM Urbanza: Nanopolimers easy the deeper penetration in complex biofils produced by multiple species. The synergist action: secondary metabolites often have improved effects when associated with nanoparticles, increasing their mixed communities against mixed mixed communities. Target specification: Nanopartices functionality allows the target's specific pathogen or tissue.

MATERIALS AND METHODS

Extraction, Isolation and purification of secondary metabolites :-

ISOLATION:

1. Powdered cinnamon is taken and mixed with 99% ethanol in ratio of 1:9 and heavily stirred in a beaker for 15-20 mins.
2. Filtered the solution and kept the filtrate (Ethanol solubilize the coumarin along with other phenols and flavins in it).
3. Phenol is removed by heating it in air oven at 60 degree celcius for time period per 5g of cinnamon for 2-3 hours.

EXTRACTION AND PURIFICATION:

1. After the ethanol solvent evaporation,the extract is now devoid of phenol and other less boiling point materials,forming blackish extracts of small dusts.
2. The blackish extract has some water soluble contents that is eluted out by mixing 10ml of distilled water and mixed them in magnetic stirrer for half an hour.
3. Then the mixture is evaporated to black dust.
4. Resultant coumarin solvent forms from black dust by mixing it with 50% ethanol mixed in mortar and pestle.
5. Now NaOH is added to make the pH to 8 to avoid precipitation.

Biochemical Characterization of Coumarin :-

TEST FOR COUMARIN:

5 3 ml of 10% NaOH □is taken and mixed in 2mg of blackened extract.

confirms the presence of

- Fade yellow colour is obtained which shows the presence of coumarin. Hence in the figure given below, yellow colour is formed which coumarin.

TEST FOR PHENOL:

- 5% Fecl3 is made on the base of excess NH4OH.
- Black colour is obtained which shows that phenol is present in the sample.

Hence in the figure given below, black colour is formed which phenol confirms the presence of in my coumarin sample.

DETECTION:

- UV-Vis Spectrophotometer: 300 nm-0.35

  240 nm-0.25

Hence my sample has given the UV range of 0.23 in 240nm in sphectrophotometer..So, Coumarin is detected.

PURITY ASSESMENT:

- 2 Thin Layer Chromatography:

Silica is powdered using mortar pestle and distilled water is added then dried into the air oven forming a thin layer. In TLC ,Rf value Retention Factor explain the distance between the sample and the solvent. So Rf factor is basically the distance travelled by defined as

the sample divided by distance travelled by the solvent.

For TLC 40% chloroform and 60% ethanol is used. The Rf value of coumarin in a 40% chloroform mobile phase for TLC is typically around 0.5-0.7.

According to my sample(coumarin)Distance

travelled by sample=3.2 cm

Distance travelled by solvent= 6.6 cm

Therefore Rf value= 3.2/6.6 cm

=0.48 cm.

The Rf value given and the Rf value obtained from my sample is same so we got to know that purity is maintained by doing the TLC.

Nano Particle Preparation of Chitosan :-

The chitosan nanoparticle doped indocyanine green(CNPs-ICG) will be prepared. Briefly,50 mg of chitosan powder(ACROS Organic uk) with a low molecular weight(1-3 kDa) will be dissolved in 50ml deionized distilled water.

- After 1 hour at room temperature,the solution will be placed on the magnetic stirringand 1% acetic acid solution will be added dropwise within 30 mins to create the homogeneous. chitosan solution.
- Sodium tripolyphosphate [TPP 0.5% (W/V), 2mL] as a crosslinking agent will be added dropwise and the compound will be stirred 30 meus.

(According to the Yongmei and Yumen study, TPP will strengthen the matrix of the CNPs.The mechanical strength of the CNPs matrix will increase by increasing the no of crosslinks formed between CNPs and TPP and these nanoparticles become stronger and

harder).

- 2.0 mg/mL ICG will then be added to the prepared chitosan solution and mixed via a magnetic mixer for 10 meus at 1000 rpm, în at pH 4.8.
- Eventually, this solution will be centrifuged at 10,000 ppm for 30mins.

- The collected sediment CNPS-ICG will be typ lyophilized for further experiments. The entire chitosan nanoparticle solution is passed through a 0.22 micron syringe filter to make sure particles below the range of 200 nm passes through the solution after which the entire solution is scanned from 200-800 nm in spectrophotometer which should give a peak concluding nanoparticles are formed.

Fig: The UV range in spectrophotometer without the coating of the nanoparticle

- Once the nanoparticles are coated with drugs again the solution is scanned through a UV Sphectrophotometer from 200-800 nm and again the peak is observed.

Now in comparision we can see the peak before and the peak after has subsequential changes.So, the after peak has the much higher wavelength or higher absorbance than the only chitosan nanoparticle which indicates the successful coating of the drug on the nanoparticles.

Minimum Inhibitory Concentration (MIC):-

The MIC (Minimum Inhibitory Concentration) is a method to find the lowest amount an of antimicrobial agent that stops the visible growth of a microorganism in a controlled setting. This method is commonly used for testing how sensitive microbes are to antibiotics. To conduct MIC testing, different concentrations of antibiotics are prepared and tested against a standardized amount of bacteria, usually in LB or on agar media.

Historically, evidence for an infectious disease causative agent was obtained by fulfilling Koch's postulates, which require the isolation of the microbe responsible for infection from the site of infection, cultivation in a laboratory, and reproduction of the initial infection by

introducing pathogen back into the host. They support the identification of microbial species present at the moment of infection by in the course of the disease. The theories 13 are based on the excluding the presence of other microorganisms assumption that secondary microbial species with low abundances play no role in inciting the disease. However, it is becoming most obvious, even to the most credulous, that most, if not all, diseases are not monomicrobial in nature and manifestation. Specifically, at this point we believe that many infections initiate—as remain—polymicrobial, 15 similar to most wound infections and CAUTI, or they initiate as

polymicrobial inoculation into the infection site.

Indeed, since it was recognized for some time, the role that polymicrobial bacterial communities can play in driving specific disease states—such as otitis media and periodontitis—is relatively well characterized. However, there is emerging consensus that some traditionally considered monomicrobial diseases, such as urinary tract infections, are also driven by other organisms present at lower titers during active infection and by the microbial consortium at the time of infection. The formation of biofilms-often protected environments in which microbial organisms can replicate-is another common feature in chronic and/or recurrent infections. For polymicrobial culture, three or four types of bacteria able to metabolize on their own on one culture are needed with no certain hurdles required. Whereas most bacteria in nature either coexist peacefully with other bacteria or are thoroughly inhibited by them due to a certain bacteriotoxin, in the laboratory environment one unique bacterium is isolated, cultivated and then introduced back with the rest. The primary purpose of testing for antibiotic activity in polymicrobial cultures is to find evidence of broad-spectrum antibiotic action of a synthesised component or phytochemical. While a certain bacteria will cause a different disease depending on the source of contamination and whether the bacterium exists in multicolonial form, it must be able to be infectious at that number. But the goal of exploiting that

component in the future is to become an antibiotic to a number of bacteria, meaning that if a drug is made with it, its effectiveness must count to be cost-effective and help the economy of medicines.

Polymicrobial Cohort Preparation

In order to make polymicrobial cultures, 2 different sets of microorganisms were made. The 3 different sets were as follows:

Set 1: Enterococcus faecalis, Listeria monocytogenes, Serratia marcescens

Set 2: Pseudomonas aeruginosa, Campylobacter jejuni, Listeria monocytogenes

Set 1 was made on the basis that both the Enterococcus and Listeria species secretes cytolysin toxin that form pores on the cell membrane of Gram-positive bacteria like Staphylococcus aureus and Lactococcus lactis. Serratia species was made a part of this set as it secretes ShlA toxin that inhibits Gram negative bacteria.

Set 2 was made on the basis that Campylobacter and Listeria survive together in livestock and are the causal agents of several diseases in animals. Pseudomonas forms biofilm in case of environmental stress conditions. Formation of biofilm helped the other two microorganisms along with Pseudomonas to survive together

Microbial colonies from plates of 2 different bacteria for each set was taken using an inoculation loop and then inoculated into liquid LB media of 60 ml and then kept in an

5 at 37°C for 24 hours. incubatorAfter the incubation period, those cultures were again

spread on specific media of those three bacteria and the microbes were allowed to grow at 37°C. Next day, each bacterial plate contained 95% of the specific bacteria which confirmed the growth of the three microbes in the poly microbial cohort. Also, staining confirmed the presence of the three microbes in the cohort. The cohort was prepared according to the survivability of the microbes with

each other and hence we checked the survivability according the gram character, toxins released and rate of competitive

inhibition.

The steps of doing MIC are as follows:

 Bacterial plate preparation-

1.Media of 45 ml(with agar) was prepared and it was heated in microwave oven

2. Waited until it cooled down properly.

3.Poured them in 5 different plates (As 2 sets of polymicrobial bacterial was used and each set has 3 different types of bacteria and 1 bacteria common in both the sets)

4.Added a drop of bacterial culture from the mother/father culture.

5.Spreading was done.

6.Sealed the plate using tape to avoid contamination and kept in incubator for the overnight growth of the bacterial culture.

 Inoculation-

1.For inoculation a LB Broth was prepared.

2. A minimal loop from the polymicrobial set of bacteriaagar 6 was isolated from the media

3.Then that loop was transferred into the LB media and kept in shaker for further mixing and the proper growth of the bacterial culture in the LB media.

- After some hours, OD was taken into the spectrophotometer and waited until it reaches 0.55-0.6 which indicates a specific bacterial growth.
- After a while when OD reached 0.6, 3 ml was kept for the standard solution and the drug(nanocoated coumarin sample) was added to the LB media to check whether the sample drug

could inhibit the bacterial growth.The dosage of drug added was calculated as (C1)(V1)=(C2)(V2) which is shown below.

- 3 different OD of the standard solution and the sample solution has been recorded in the interval of 1 hour to see the difference of 11 in the sample solution growthcomparing it with the standard solution.

The polymicrobial set of bacteria thatset I 2 has been used for are- Enterococcus faecalis, Listeria monocytogenes, Serratia marcescens.

C1(Initial conc) = 10,500 µg/ml V1(

Drug volume) = ?

C2(Final conc) = 100 µg/ml

V2 (Standard solution) = 3ml

(C1)(V1)=(C2)(V2)

10,500*V1=100*3

V1=28.5 µl

The polymicrobial set of bacteria thatset has been used for II are- Pseudomonas aeruginosa, Campylobacter jejuni, Listeria monocytogenes.

C1(Initial conc) = 10,500 µg/ml V1(

Drug volume) = ?

C2(Final conc) = 192.5 µg/ml

V2 (Standard solution) = 3ml

(C1)(V1)=(C2)(V2)

10,500*V1=192.5*3

V1=55 µl

Half Maximal Inhibitory Concentration(IC50) :-

Basically IC50 is defined as the how much the drug could inhibit a specific biological reaction by 50%.In case of IC50 value, the drug is more effective because it inhibits the growth at half concentration. Like MIC, in IC50 too 2 sets of polymicrobial bacteria are used for the dosage of drug to show the inhibition at 50% concentration. The dosage has been calculated as (C1)(V1)=(C2)(V2) as shown below-

For IC 50 (Set I)-

C1(Initial conc) = 10,500 µg/ml V1(

Drug volume) = ?

C2(Final conc) = 220 µg/ml

V2 (Standard solution) = 3ml

(C1)V1=(C2)(V2)

10,500*V1=220*3

V1=62.8 µl

For IC 50 (Set II)-

C1(Initial conc) = 10,500 µg/ml V1(

Drug volume) = ?

C2(Final conc) = 260 µg/ml

V2 (Standard solution) = 3ml

(C1)V1=(C2)(V2)

10,500*V1=260*3

V1=78 µl

Zone Of Inhibition(ZOI)-

It is defined as the area where the bacteria or fungus could not grow due to a presence of drug that inhibites the growth rate of a particular bacteria or fungus We have done ZOI by using polyset 1 MIC bacteria .

Enterococcus faecalis, Listeria monocytogenes, Serratia marcescens The steps of

doing Bacterial ZOI are as follows:

- First we prepared LB agar media and heated .
- Then poured the agar media into a petri dish.
- Then wait for sometime so that it cool down properly.
- Then we make a well in the middleadd 2 of the plate and polyset bacteria culture approx 100µl.
- We kept the plates in incubator for 24hours
- Next day we saw the ZOI in the plate
- Then the diameter of the ZOI was measured

Also ZOI emphasizes on the fungi Aspergillus niger.So, fungal ZOI is a clear area around an antifungal drug on a petri dish, showing where fungal growth is stopped. This zone shows how well the drug works. Larger zones mean stronger antifungal activity.

The steps of doing fungal ZOI are as follows:

- At first PDA media was prepared
- Then inoculated with the fungi Aspergillus niger.
- A well was cut using a tip in that petri dish and in that well 100 µl of the drug sample(nanocoated coumarin sample) was added.
- Incubated for 24 hours
- The diameter of ZOI was measured around the well. The larger ZOI indicates the greater antifungal activity.

RESULT AND DISCUSSION

Table 1: The result of Set I Bcterial MIC.The dosage given is compared to the standard.

Starting OD=0.59

Standard Dosage	
0.66	
0.58	
0.79	
0.58	
0.84	
0.61	

Fig : The graph of MIC polymicrobial set I..

Table 2: The result of Set I MIC.The dosage given is compared to the standard

Starting OD=0.61

Standard	Dosage
0.72	0.62
0.76	0.64
0.80	0.67
0.82	0.65

Fig : The graph of MIC polymicrobial set II.

Table 3: The result of Set I IC50.The dosage given is compared to the standard.

Starting OD=0.54

Standard

Dosage

0.50

0.44

0.50

0.38

0.45

0.33

Fig : The graph of IC50 polymicrobial set I.

Table 4: The result of Set II IC50.The dosage given is compared to the standard.

Starting OD=0.54

Standard

Dosage

0.53

0.47

0.55

0.43

0.49

0.38

Fig : The graph of IC50 polymicrobial set II.

Table 5: The result of Fungal MIC.The dosage given is compared to the standard.

Starting OD=0.61

Standard

Dosage

0.67

0.58

0.72

0.62

0.76

0.65

Fig: The graph of Fungal MIC..

Table 6: The result of Fungal IC50.The dosage given is compared to the standard.

Starting OD=0.56

Standard Dosage	
0.50	0.46
0.50	0.44
0.45	0.38

Fig: The graph of Fungal IC50.

Fig:- A clear Circular Zone Of Inhibition(Fungal) is observed around the disc above in the figure. It means that the dosage of the coated sample (coumarin) could inhibit the fungal growth in the PDA agar plate.

Fig:- A clear Circular Zone Of Inhibition(Bacterial) is observed around the disc above in the figure. It means that the dosage of the coated sample (coumarin) could inhibit the bacterial growth in the LB agar plate

CONCLUSION

It could be concluded from the above data and graphs that Coumarin has a antibacterial property present in them and couldand 6 inhibit the growth of bacteria fungi.It works against both gram positive and gram negative bacteria.Coumarin is a potent natural antimicrobial agent.Coumarin also gives development of many alternative antimicrobial therapies.So, overall this study lead us to the path of many new upcomings of the advantages of coumarin as an important antimicrobial agent and also encourages continued research in pharma and clinical development.

Sources

1	https://www.jetir.org/papers/JETIR2203035.pdINTERNETf	5%
2	https://microbeonline.com/thin-layer-chromatographyINTERNET /	1%
3	https://www.jetir.org/papers/JETIR2203035.pdf#:~:text=Coumarins comprising of intertwined benzene and α-pyrone rings,seeds, roots, and leaves of many plant species. INTERNET	<1%
4	https://acknowledgement.in/projectINTERNET /	<1%
5	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8424233INTERNET /	<1%
6	https://pmc.ncbi.nlm.nih.gov/articles/PMC8541629INTERNET /	<1%
7	https://www.sciencedirect.com/science/article/pii/B9780128037973000011#:~:text=The derivativefrom greens oplantsf coumari. n usually occur as secondary,identified from natural sources, especially	

	INTERNET <1%	
8	https://link.springer.com/article/10.1007/s00044-016-1519-INTERNET 9 <1%	
9	https://www.chemistrywithdrsantosh.com/2023/05/Rf-value-calculation-significance-and-affecting-factors.html INTERNET <1%	
10	https://www.clinicalmicrobiologyandinfection.com/article/S1198-743X(14)64063-5/fulltexINTERNET t <1%	
11	https://biologynotesonline.com/chromatography-principle-types-applicationsINTERNET / <1%	
12	https://pmc.ncbi.nlm.nih.gov/articles/PMC7913839INTERNET / <1%	
13	INTERNEThttps://thisvsthat.io/column-chromatography-vs-thin-layer-chromatography <1%	
14	https://www.ijsr.net/archive/v11i7/SR22701191814.pdINTERNET f <1%	
15	https://www.sciencedirect.com/science/article/pii/S002228361630144INTERNET 9 <1%	
16	https://link.springer.com/article/10.1134/S106816202405010INTERNET 8 <1%	

17 https://chemicalmatter.com/coumarin/#:~:text=Coumarin is a naturally occurring compound found in,commonly used in perfumes, cosmetics, and food flavorings.
INTERNET
<1%

---

18 INTERNET https://simplesolvents.com/simple-solvents-blog/pharmaceutical-grade-solvent-uses/
<1%

EXCLUDE CUSTOM MATCHES	ON
EXCLUDE QUOTES	OFF
EXCLUDE BIBLIOGRAPHY	OFF